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INHIBITION OF TUMOR GROWTH BY SYSTEMIC TREATMENT WITH THROMBOSPONDIN-1 PEPTIDE MIMETICS

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Many normal human cells produce thrombospondin-1 (TSP-1), a potent antiangiogenic protein that promotes vascular quiescence. In various organ systems, including the brain, breast and bladder and in fibroblasts, TSP-1 secretion is reduced during tumorigenesis, thereby allowing induction of the vigorous neovascularization required for tumor growth and metastasis. Full-length and short TSP-1–derived peptides inhibit angiogenesis by inducing endothelial cell apoptosis and thus disrupting the vasculature of the growing tumor. CD36 expressed on the surface of endothelial cells functions as the primary antiangiogenic receptor for TSP-1. A D-isoleucyl enantiomer of a TSP-1 heptapeptide specifically inhibits the proliferation and migration of capillary endothelial cells. DI-TSP, an approximately 1 kDa capped version of this peptide, is also antiangiogenic *in vitro*, with a specific activity approaching that of the 450 kDa parental molecule. Here, we show that DI-TSP delivered systemically dose-dependently inhibits the growth of murine melanoma metastases in syngeneic animals and that its more soluble isomer, DI-TSPa, similarly blocks the progression of primary human bladder tumors in an orthotopic model in immune-deficient mice. Like intact TSP-1, these peptide mimetics had no effect on cancer cells growing *in vitro* but markedly suppressed the growth of endothelial cells by inducing receptor-dependent apoptosis. Antibodies raised against CD36 blocked the ability of peptides to induce apoptosis in endothelial cells but had no effect on tumor necrosis factor-α–induced apoptosis. *In vivo*, the peptide mimetics were associated with a significantly reduced microvessel density and increased apoptotic indices in both the endothelial and tumor cell compartments. Such short peptides targeted to a specific antiangiogenic receptor, potent and easy to synthesize, show great promise as lead compounds in clinical antiangiogenic strategies.

Key words: *tumor angiogenesis; thrombospondin-1; peptide mimetics; bladder cancer; melanoma*

Solid tumors and their metastases are critically dependent on neovascularization for progressive growth. If vessels cannot form, tumors remain dormant at a threshold size of several millimeters in diameter.[1,2] Whether or not vessels develop is determined by the balance between inhibitors of angiogenesis and angiogenic stimuli present within a given tissue microenvironment. In most healthy adult tissues, inhibitors predominate, favoring vascular quiescence, while in many disease states the situation is reversed, with a prevalence of inducers triggering capillary remodeling and angiogenesis.[3,4] During tumorigenesis, downregulation of secreted antiangiogenic mediators is often a key step in the development of an angiogenic phenotype (reviewed in refs. 3–5).

Blocking tumor progression by restoring the physiologic antiangiogenic environment was first proposed as an anticancer therapy by Folkman[6] and has been extensively validated in animal models.[7–13] One potential advantage of this approach is that it targets genetically stable endothelial cells rather than unstable tumor cells, so resistance to therapy is less likely to develop.[14] A variety of naturally occurring inhibitors of angiogenesis have been discovered and many of them hold particular promise for cancer therapy as they can block angiogenesis induced by the complex mixtures of proangiogenic factors produced by human tumors.

One such natural inhibitor is thrombospondin-1 (TSP-1), a large homotrimeric protein composed of multiple distinct structural and functional domains that are responsible for its diverse biologic functions (reviewed in refs. 15–17). Downregulation of TSP-1 plays a critical role in the angiogenic switch in several tumor types, including bladder cancer, breast cancer, glioblastoma and fibrosarcoma.[18–20] The antiangiogenic effect of TSP-1 is mediated by the CD36 receptor, which triggers a signaling cascade that leads to apoptosis in activated endothelial cells and, thus, to the collapse of tumor vessels.[16,21] Although the 450 kDa TSP-1 macromolecule can diminish tumor growth through its effects on the tumor vasculature,[20,22,24] its use in humans is not seriously contemplated because of its size, difficulty in producing large-scale preparations and concerns about side effects that might result from its multiple other biologic functions.[24–26] Small, TSP-1–derived peptide mimetics that can be targeted to a specific receptor provide an attractive alternative.

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Small peptides from the preprocollagen homology region and from the properdin repeats of TSP-1 also inhibit angiogenesis *in vitro*, using the same CD36-dependent pathway as the parental molecule.[16] Unfortunately, these short peptides are at least 1,000 times less active than intact TSP-1,[27,28] probably because of their high degree of conformational freedom when not constrained as part of the larger protein. Remarkably, one of these peptides, corresponding to a portion of the second of the type 1 repeats, when stabilized by replacing 1 of 3 L-amino acid residues with a D enantiomer, becomes almost as active as the parental molecule.[29] One such 7–amino acid TSP-1 peptide enantiomer, containing D-isoleucine, inhibits endothelial cell migration with an ED_{50} of 7.1 nM.[27] A modified N- and C-terminally capped version of this peptide (MW approx. 1 kDa), in which only a single additional L-amino acid is changed from that found in TSP-1, is referred to as DI-TSP (see Material and Methods).[27] This peptide displaces radiolabeled TSP-1 from cells expressing CD36 on their surface, inhibits endothelial cell migration *in vitro* at nanomolar concentrations and blocks corneal neovascularization *in vivo* when administered systemically.[27] Other small antiangiogenic peptides derived from the second of the type 1 repeat regions of TSP-1 inhibited breast tumor growth in immune-deficient mice, but their mechanism of action and effect on tumor angiogenesis were not defined.[30,31]

Here, we show that systemic administration of relatively low doses of DI-TSP or its more soluble isomer, DI-TSPa, blocks the progression of an aggressive human primary bladder cancer and significantly inhibits lung metastases in a syngeneic mouse melanoma model, both *via* inhibition of angiogenesis. The effectiveness of these CD36-targeted peptides in 2 distinct tumor types that depend on different subsets of angiogenic stimuli argues strongly for their clinical utility and demonstrates the universality of a single antiangiogenic strategy against cancer.

MATERIAL AND METHODS

Animals

Athymic male nude mice, 6–8 weeks of age, were purchased from Charles River (Wilmington, MA) and kept under pathogen-free conditions at Northwestern University animal facilities according to NIH and Northwestern University Center for Experimental Animal Research guidelines. Female C57/Bl6 mice, 4–6 weeks of age, were purchased from Harlan (Minneapolis MN).

Cells

The aggressive bladder cancer cell line 253J, variant B-V[32] was maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine and penicillin. The metastatic, nonpigmented melanoma cell line B16F10, used for the lung colonization assay, was a generous gift of Dr. I.J. Fidler (M.D. Anderson Cancer Center, University of Texas, Houston, TX) and maintained in RPMI or MEM with 10% serum, nonessential amino acids and 2 mM glutamine. Bovine adrenal capillary endothelial cells, BP10T8 (a kind gift of Dr. J. Folkman, Children's Hospital, Harvard Medical Center, Boston, MA), were grown on gelatinized surfaces in DMEM supplemented with 2% glutamine, 100 μg/ml endothelial cell mitogen (R&D Systems, Minneapolis, MN) and 10% donor calf serum and used at passage 14. All cells were incubated at 37°C at 7% CO_2.

Peptides

DI-TSP (Abbott, North Chicago, IL) is a capped nonapeptide based on the linear TSP-1 heptapeptide sequence from within the second properdin repeat, GVITRIR, containing a D-isoleucyl substitution for the first L-isoleucine[29] and in which the internal Arg residue is replaced by Nva. The sequence of DI-TSP is N-Ac-Gly-Val-D-Ile-Thr-Nva-Ile-Arg-Pro-NHEt. Thus, 5 of 7 contiguous amino acids are exactly equivalent in identity and relative position to the TSP-1 sequence. In DI-TSPa, the D-Ile residue is replaced by D-allo-Ile. Both peptides display similar *in vitro* activity against endothelial cells. Bowes melanoma cells transfected with CD36 bound more europium-labeled TSP-1 than the parental line. Both DI-TSP and DI-TSPa at 1 nM completely displaced this excess binding of 100 pM Eu-TSP-1, as did a 40-fold excess of unlabeled TSP-1 (data not shown). Both peptides were synthesized by solid-state methods using 9-Fluorenyl-methoxy-carbonyl (FMOC)-protected amino acids and purified by reverse-phase HPLC as described previously.[26] Lyophilized peptides were readily dissolved in either sterile PBS or sterile 5% dextrose, in which solvents underwent no measurable degradation during 1 week at ambient temperature.

Orthotopic implantation of tumor cells

Cultures of 253J B-V cells (50–60% confluent) were replenished with fresh medium and harvested within 24 hr by brief trypsinization and their viability was determined by trypan blue staining. Single-cell suspensions with no less than 95% viable cells were used. Mice were anesthetized with methoxyflurane and a lower midline incision was made, followed by exteriorization of the bladder. Under a dissection microscope, 5×10^5 tumor cells were injected into the wall of the bladder through a 27-gauge needle. Localized swelling was scored as a successful injection. Incisions were closed with metal clips and the animals subsequently treated and observed for 31 days. Animals were then sacrificed and the bladders removed, weighed, measured and processed for further analysis. Tumors harvested at autopsy were divided into fragments and fixed in 10% buffered formalin (Sigma, St. Louis, MO) or in OCT compound (Sakura, Torrance, CA) to be snap-frozen in liquid nitrogen. Sections were counterstained with hematoxylin and eosin. Eight animals were included in each control and treatment group and all experiments were repeated 3 times.

Lung colonization assay

Lung colonization assay was performed as previously described.[29] B16F10 cells were grown to 80–90% confluence, harvested and resuspended in saline; 10^5 cells in 0.1 ml saline were injected into the tail vein of C57/Bl6 mice through a 27-gauge needle. After 21 days, animals were sacrificed and the lungs harvested, weighed and fixed overnight in Bouin's solution.[29] Lungs were embedded in paraffin for further analysis. Five animals were included in each of the control and treatment groups and all experiments were repeated 3 times. All metastases visible on the surface of excised lungs were counted and in each case 50 tumors were randomly selected and measured to estimate mean tumor size.

Systemic treatment

Following surgery or tail-vein injections, mice were mixed and distributed randomly into treatment groups. Systemic therapy started on day 3 postimplantation for the bladder cancer model and 24 hr postinjection for the melanoma model. Mice (groups of 8 for bladder cancer and 5 for melanoma) received peptides at increasing concentrations or vehicle saline twice a day into the peritoneal cavity. Animals inoculated with melanoma cells received DI-TSP and those with bladder tumors were treated with DI-TSPa, which had just become available.

Microvessel density

Microvessel density was evaluated by immunohistochemistry performed on frozen sections using a rabbit antimouse monoclonal antibody (MAb) against CD31 (Pharmingen, San Diego, CA) as described.[32] Areas of the highest capillary density (hot spots) were identified and counted under a light microscope in 400× high-powered fields, using 3 areas per section. Vessels were identified according to the criteria established by Weidner *et al.*[33]

Detection of endothelial cell apoptosis

Sections were stained for CD31 as above. However, R-phycoerythrin–conjugated polyclonal donkey antirat Ig-specific polyclonal antibodies (Becton Dickinson, Los Angeles, CA) were used

as the secondary antibody and the same sections were subsequently stained for nucleosomal DNA fragmentation using the ApopTag Fluorescein Apoptosis Detection Kit (Intergen, Purchase, NY) following the manufacturer's instructions. Images were analyzed by fluorescent microscopy and superimposed using Corel Photo-Paint software (Corel Corp., Ottawa, Ontario, Canada). The percentage of apoptotic endothelial cells (yellow) when compared to total endothelial cells (red) was calculated. In each case, at least 250 total cells were counted.

Assessment of proliferative index

Tumor cells were stained using the proliferations cell nucleur antigen (PCNA) Kit (Zymed, San Francisco, CA) with a microwave antigen retrieval step added according to the manufacturer's instructions.

In vitro *growth studies*

B16F10 melanoma, 253J B-V bladder cancer and BP10T8 endothelial cells were plated in complete media in 96-well plates at 5×10^2 cells/well for cancer cells or 1×10^3 cells/well for endothelial cells. On the following day, cancer cells were replenished with medium supplemented with 2% serum and endothelial cells, with serum-free medium plus 0.1% BSA and 20 ng/ml basic fibroblast growth factor (bFGF). DI-TSP was added where indicated. Media and peptides were changed daily. Proliferation was measured using the CellTiter 96 Kit (Promega, Madison, WI) as recommended by the manufacturer. Each condition was repeated in triplicate and the data are reported as absorbance at 490 nm.

Evaluation of apoptosis in vitro

Cells were plated on gelatinized glass coverslips in 24-well tissue culture plates at 5×10^4 cells/well, treated with the indicated compounds in low (0.2%) serum, fixed in 1% buffered paraformaldehyde, stained using the ApopTag Direct Kit (Intergen) following the manufacturer's instructions and counterstained with propidium iodide; then, the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells was calculated. At least 500 cells were counted per test condition. Anti-CD36 MAb FA6-152 (Immunotech, Miami, FL) was added where indicated.

Measurement of CD36 receptor levels

All cells were analyzed by flow cytometry as previously described.[15] Mouse melanoma cells, bladder cancer cells and endothelial cells were plated into 60 mm tissue culture dishes, harvested at 80% confluence in 2% EDTA in PBS and washed twice with 2% FCS in PBS. Cells were then incubated for 45 min at 4°C with FITC-conjugated anti-CD36 MAb SMØ (Ancell, Bayport, MN) or with control IgM (Sigma) at 5 μg/ml and then analyzed using a Coulter Beckman (Fullerton, CA) flow cytometer.

Statistical analysis

Mean values of microvessel density, tumor volume, tumor number and *in vitro* parameters were compared using Student's *t*-test. A 2-sided p value < 0.05 was considered statistically significant.

RESULTS

Systemic treatment with TSP peptide mimetics

To determine whether antiangiogenic TSP-1 peptide mimetics could replicate the antitumor activity of the whole TSP-1 molecule, both DI-TSP and DI-TSPa, D-Ile–containing enantiomers of a short antiangiogenic peptide derived from the second repeat in the TSP-1 properdin homology domain,[27] were used to treat tumor-bearing animals.

Tail vein injection was used to implant highly metastatic syngeneic murine melanoma cells (B16F10) into wild-type mice, which were then treated with DI-TSP. As reported previously for intact TSP-1,[29] DI-TSP administered at 10, 50 and a 100 mg/kg twice daily caused a marked dose-dependent decrease in both the number and size of visible tumor colonies formed in the lungs of mice (Fig. 1*a–c*). Colonies detectable by the naked eye were rarely seen in the lungs of DI-TSP–treated animals. Quantification with the aid of a dissection microscope revealed a 5.5-fold decrease in the number of metastases and a more than 6-fold decrease in the mean diameter of surface colonies after 15–21 days of peptide treatment (Fig. 1*b,c*). While the number of macroscopic lung colonies was diminished in peptide-treated animals, the number of microscopic tumors remained substantial, pointing to a primary restriction on tumor size posed by the antiangiogenic DI-TSP (Fig. 1*a*).

Surgery was then used to implant human bladder cancer cells (253J B-V) orthotopically into immune-deficient mice, which were subsequently treated with DI-TSPa, a more soluble enantiomer of DI-TSP. The 2 peptides have the same molecular formula and weight, have almost identical activity against endothelial cells *in vitro* and differ only in their chirality at a single carbon (see Material and Methods). The serum half-life of DI-TSPa in rodents is slightly longer than that for DI-TSP (22.7 *vs.* 13.2 min after i.v. injection of 4.5 mg/kg into normal, healthy animals). Tumor take for bladder tumors was 100% in both peptide-treated and control animals. Mean tumor volume decreased approximately 4-fold in the group treated with DI-TSPa at 27 mg/kg twice daily (Fig. 1*d*). Treatment with increasing doses of DI-TSPa yielded a dose-dependent decrease of mean tumor volume (Fig. 1*e*).

DI-TSP and DI-TSPa selective blockade of capillary endothelial cell growth

To examine the mechanism underlying the ability of DI-TSP and DI-TSPa to block tumor progression, cultured microvascular endothelial cells, 253J B-V bladder cancer cells and B16F10 melanoma cells were treated *in vitro* with DI-TSP or DI-TSPa and the effects of the peptides on cellular proliferation and apoptosis measured. Both DI-TSP and DI-TSPa suppressed the growth of cultured bovine capillary endothelial cells in a dose-dependent manner with nearly complete growth suppression observed at concentrations sufficient to inhibit endothelial cell migration (100 ng/ml).[27] This effect could be seen by direct cell counts and using an indirect MTT proliferation assay (Fig. 2*a* and data not shown). In contrast to the sensitivity displayed by endothelial cells, neither of the tumor cell lines was inhibited by the peptides at 100 ng/ml (Fig. 2*b,c*), suggesting that the antitumor activity of these peptides was due to inhibition of endothelial cell function.

Both DI-TSP and its variant DI-TSPa induced apoptosis in cultured capillary endothelial cells (Fig. 2*d,e*). The levels of apoptosis induced by both TSP-1 peptides were comparable to those induced in these cells by intact TSP-1.[21,23] Peptide-induced apoptosis was prevented by the FA6-152 MAb, which blocks access to CD36, the primary antiangiogenic receptor for intact TSP-1, but not by an isotype-matched control antibody (Fig. 2*d*). The effect of FA6-152 was specific because it failed to block endothelial cell apoptosis induced by tumor necrosis factor-α (Fig. 2*e*). Although both 253J B-V and B16F10 cells expressed levels of cell-surface CD36 sufficient to register by FACS analysis, apoptosis could not be induced in either line by TSP-1 or its peptides (data not shown).

TSP-1 peptide mimetics and tumor vascularity

To determine if the TSP-1–derived peptides that were so effective as antiangiogenic agents *in vitro* did indeed inhibit neovascularization *in vivo*, the tumor vasculature was visualized using antibodies against the endothelial cell marker CD31 (platelet endothelical cell adhesion molecule [PECAM-1]), which effectively stains murine vessels.[33] Tumor microvessel density was significantly reduced in peptide-treated animals (orthotopic bladder cancer model) compared to controls (Fig. 3). Quantitative analysis revealed a drop in capillary density from 48.8 ± 1.7 to 22.6 ± 1.1 vessels per 400× field ($p < 0.05$), yielding a 2-fold decrease in tumor-associated vessels following treatment with DI-TSPa at a dose of 27 mg/kg bid.

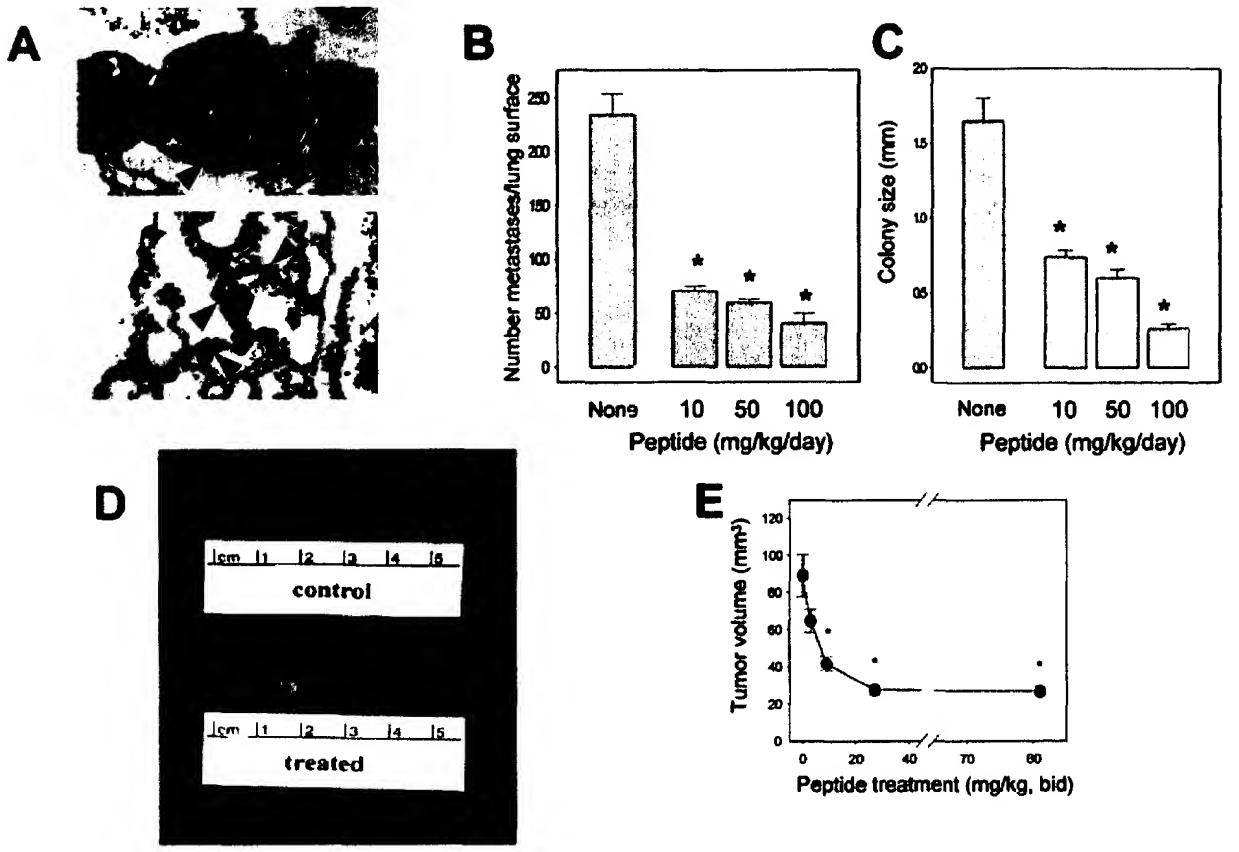

FIGURE 1 – TSP-1–derived peptide mimetics slowed the growth of tumors in the bladder and lung. *(a–c)* C57/Bl6 mice were given tail vein injections of nonpigmented B16F10 melanoma cells and treated with increasing doses of DI-TSP; 21 days postinjection, lungs were extracted and grossly examined for the presence of tumor colonies. *(a)* Histology of lung colonies from control (top panel) and treated (lower panel) animals (×100). The number of surface lung tumors *(b)* and the average lung colony size *(c)* decreased in proportion to increasing peptide dose. SEM are indicated. *Significantly different from untreated control ($p \leq 0.05$). *(d,e)* Athymic nude mice were injected orthotopically with human bladder cancer cells (253J B-V), treated with DI-TSPa starting at day 3 after surgery and sacrificed at day 31. *(d)* Dissected tumors from treated animals were decreased in size compared to controls. *(e)* Increasing concentrations of peptide resulted in decreased tumor volumes.

Decreased vessel density in peptide-treated tumors and endothelial cell apoptosis

Seeking a link between endothelial cell apoptosis *in vitro* and the previously observed decrease in tumor growth and neovascularization caused by TSP-1 peptide mimetics, sections of melanoma lung tumors and orthotopic bladder tumors were stained to colocalize the endothelial cell marker PECAM-1 and apoptotic cells detected by TUNEL staining (Fig. 4). The percentage of endothelial cells undergoing apoptosis was increased after treatment with DI-TSP or DI-TSPa by approximately 3.3- and 4.6-fold, respectively ($p < 0.03$ and < 0.01, respectively).

Proliferation and apoptotic indices were compared between orthotopic bladder tumors treated with DI-TSPa at 27 mg/kg bid and control animals. This analysis revealed a statistically significant 1.9-fold increase in tumor cell apoptosis ($p < 0.003$) after peptide treatment, whereas proliferation indices revealed by PCNA staining remained stable (Table I).

DISCUSSION

In this study, we demonstrate that simple peptide mimetics of TSP-1 are effective as *in vivo* antitumor agents in 2 distinct tumor models, primary bladder cancer and metastatic melanoma. Both bladder cancer and melanoma rely for angiogenesis on a complex milieu of secreted cytokines and growth factors that are only partially overlapping. Functional studies show that in bladder cancer vascular endothelial growth factor (VEGF), but not FGF-2, is the principal angiogenic factor,[18] whereas in melanoma VEGF is necessary but not sufficient for neovascularization and FGF-2 is both necessary and sufficient.[35–37] Just as the *in vitro* effects of TSP-1 and its peptides are independent of the inducer used,[27,28] the TSP-1 peptide mimetics retain their efficacy *in vivo* against complex and potent mixtures of various inducers of tumor angiogenesis.

TSP-1–derived peptides were highly potent in both immune-deficient and immune-competent models, suggesting that their antitumor effects were independent of the immune response of the host. Inhibition of tumor progression by DI-TSP and DI-TSPa appeared to be caused primarily by direct effects on endothelial cells for it was accompanied by a 2-fold reduction in microvessel density and a 3- to 4-fold increase in the number of apoptotic endothelial cells within the tumor. Our observation that the tumor cell mitotic rate was not diminished suggests that the peptides had no direct toxic effects on the tumor cells *in vivo*. It is likely that the increased apoptotic index in tumor cells in treated animals was a secondary effect related to a restricted blood supply. Other antiangiogenic agents that are specific for endothelial cells induce tumor regression in a similar fashion, primarily by stimulating tumor cell apoptosis without significantly diminishing mitotic rate.[38] Mechanistically, our data suggest that DI-TSP and DI-TSPa exert their antiangiogenic effects through CD36, similar to the parental molecule. Both peptides displaced labeled TSP-1 from CD36 and antibodies to CD36 abrogated their ability to induce apoptosis in endothelial cells.

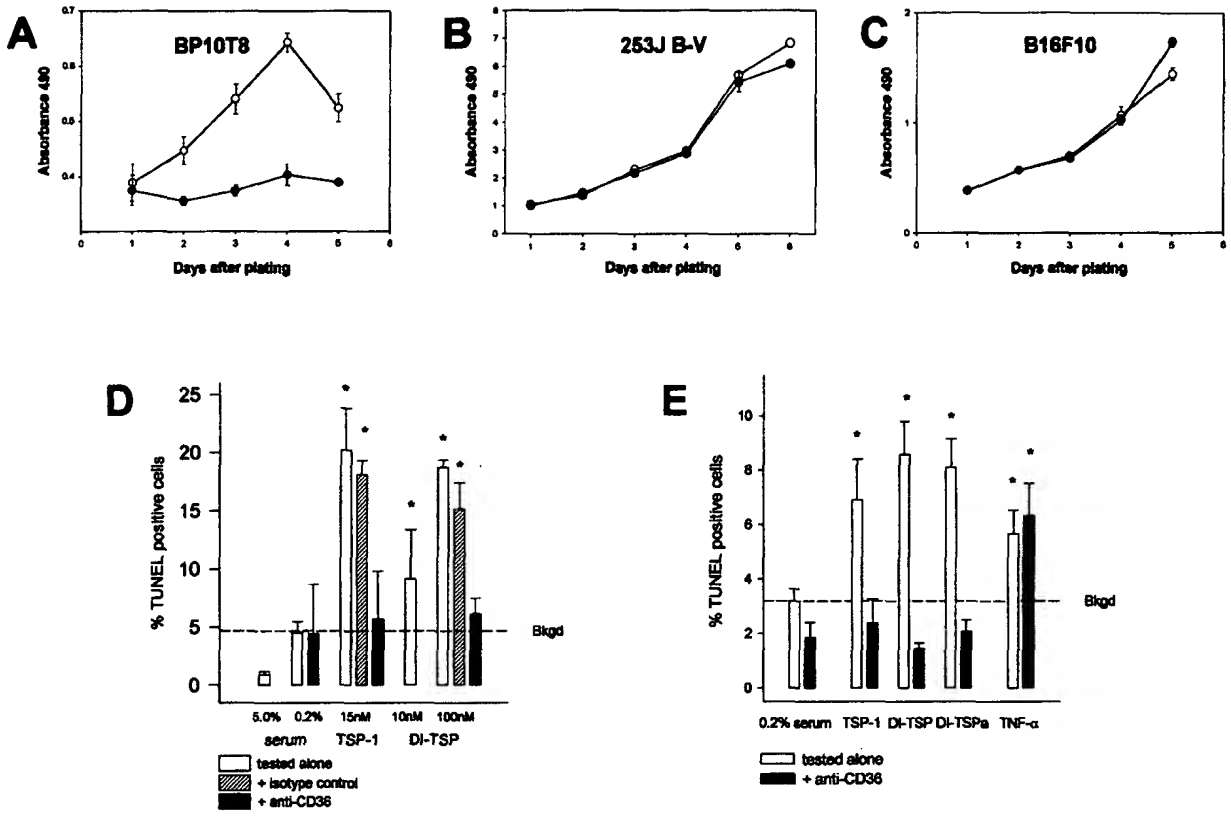

FIGURE 2 – DI-TSP and DI-TSPa inhibited the growth of and induced apoptosis in, capillary endothelial cells. Peptide mimetics were added at 100 ng/ml to parallel cultures of bovine endothelial cells *(a)*, bladder cancer 253J B-V cells *(b)* or mouse melanoma B16/F10 cells *(c)*; proliferation was assessed using an MTT assay kit (Promega). Peptide-treated cells (solid circles) were compared to untreated control cells (open circles). Data are for DI-TSP; nearly identical results were obtained with DI-TSPa (data not shown). *(d)* Induction of apoptosis in capillary endothelial cells by TSP-1 and DI-TSP alone (open bars) in the presence of anti-CD36 blocking antibodies (FA6-152, 2 μg/ml) (solid bars) or isotype-matched control antibodies (hatched bars) was compared to baseline apoptosis levels (0.2% serum). FA6 blocked apoptosis by both TSP-1 and DI-TSP. *(e)* Capillary endothelial cell apoptosis induced by TSP-1, DI-TSP and DI-TSPa. All 3 compounds caused apoptosis to an extent similar to tumor necrosis factor-α (30 ng/ml in the presence of 1 μg/ml cycloheximide), which was used as a positive control.

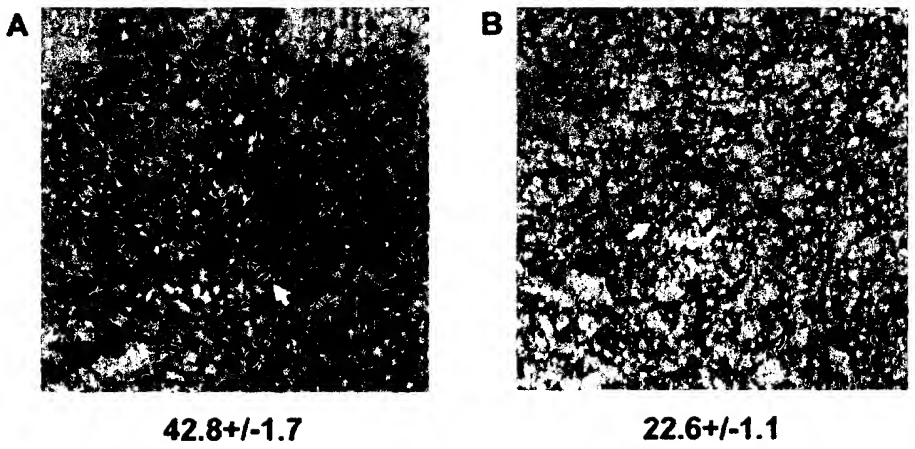

FIGURE 3 – Tumor microvessel density was decreased in animals treated with DI-TSPa. Bladder tumors from control animals *(a)* and from those that received DI-TSPa at a dose of 27 mg/kg bid *(b)* were removed, snap-frozen and stained for PECAM-1/CD31 to visualize microvessels. Mean microvessel density values with SEs are indicated below each panel. Arrows indicate representative microvessels. Magnification × 200.

The peptides proved efficacious *in vivo* despite data suggesting relatively quick clearance. The half-lives of DI-TSP and DI-TSPa in the serum of normal rodents were only 13.2 and 22.7 min, respectively. In recognition of this, twice-daily administration was used for these experiments, yielding more reproducible results than our preliminary studies, which incorporated once-daily dosing

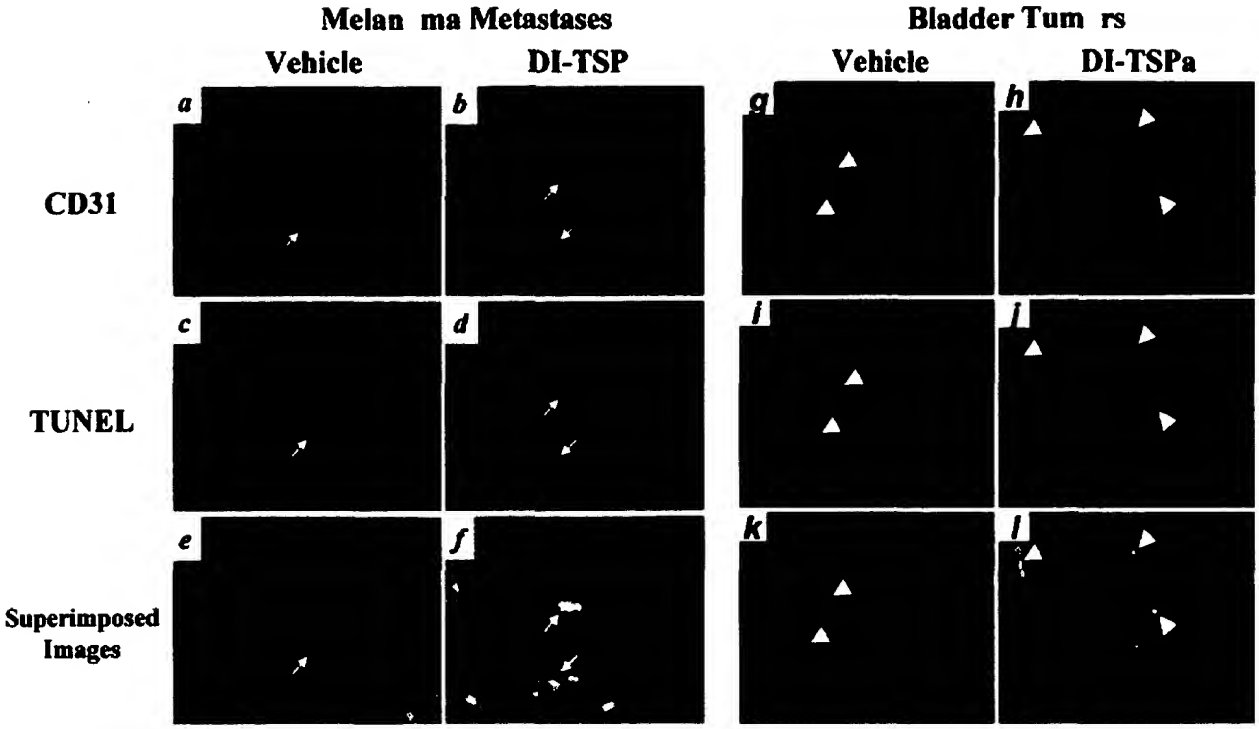

FIGURE 4 – Endothelial cell apoptosis was increased in experimental melanoma metastases and bladder tumors treated with DI-TSP or DI-TSPa. Tumors from peptide-treated and control animals were harvested, fixed and stained for endothelial cells using primary antibodies against CD-31 and phycoerythrin-conjugated secondary antibody (red fluorescent stain, upper panel). Cells undergoing apoptosis were detected using the TUNEL assay with FITC-labeled fluorescent nucleotides (Apoptag Direct Kit; green stain, middle panel). An overlay of images identified endothelial cells undergoing apoptosis (yellow, bottom panel). *(a–f)* Melanoma tumors in the lung treated daily with DI-TSP at 50 mg/kg *vs.* controls; *(g–l)* orthotopic bladder tumors treated with DI-TSPa at 27 mg/kg bid *vs.* controls. Arrows indicate representative microvessels (*a,b,g,h*), apoptotic cells (*c,d,i,j*) and apoptotic endothelial cells (*e,f,k,l*). Magnification × 400.

TABLE I – PROLIFERATIVE AND APOPTOTIC INDICES IN TUMOR CELLS FROM PEPTIDE-TREATED *VS.* CONTROL ANIMALS

Analysis of tumor cell compartment	Controls	Peptide-treated animals[1]
Proliferative index[2]	16.2 ± 0.55	15.6 ± 1.51
Apoptotic index[3]	9.4 ± 1.5	18.0 ± 2.7

[1]Orthotopic bladder cancer model with animals treated with DI-TSPa at a dose of 27 mg/kg bid or with saline (controls).–[2]Mean percentage of tumor cells staining positive for PCNA ± SD.–[3]Mean percentage of tumor cells staining positive for apoptosis ± SD.

(data not shown). In addition, the peptides were deliberately used at much higher doses than required to yield antiangiogenic levels in serum so that a systemic antiangiogenic state could be maintained for several half-lives after each i.p. injection. Sequestration of the peptide within the tumor bed due to binding by CD36 expressed by the activated tumor vasculature could also contribute to the efficacy observed, but at present this hypothesis has not been adequately investigated.

Both active peptides contained the motif GVXXXXR, which is also found in 2 other antiangiogenic peptides derived from TSP-1: Col-I from the procollagen region and Mal-III from the third properdin repeat.[28] This motif also appears in most other TSP-1–related peptides or proteins with antiangiogenic activity, including TSP-2,[39,40] BAI1–3,[41] ADAMTS-1 and METH-1 and -2.[42,43] However, when a peptide containing this sequence was synthesized from the second properdin repeat Mal-II,[27] it was not active unless it contained a D-amino acid at specific positions. It is not yet clear whether this D-amino acid directly participates in receptor binding or merely enhances the active conformation of an otherwise unstable peptide. Iruela-Arispe and colleagues[30,31] have described other antiangiogenic peptides derived from the type 1 repeats of TSP-1 that contain the putative CD36 binding domain. These peptides, one of which contains the consensus sequence CSVTCG, can inhibit breast tumor growth in animal models but, again, only if D-reverse analogues are included in the structure. The effect of these peptides on the tumor vasculature and their dependence on CD36 was not reported, so other mechanisms of action cannot be excluded.

Although CD36 mediated the antiangiogenic apoptotic effects of our peptide mimetics on endothelial cells, its presence on tumor cells was not sufficient to make them sensitive. The small amount of CD36 expressed on tumor cells displays the epitope essential for recognition by antibodies, but it may not be functional. Alternatively, either these cells may lack the intracellular machinery necessary to execute CD36-dependent apoptosis or this pathway may be masked or overruled by constitutive activation of survival pathways, which commonly occurs in cancer cells.[44]

Our data demonstrate inhibition of invasive bladder cancer in a preclinical model through the use of targeted and specific antiangiogenic therapy. Using the same orthotopic model of bladder cancer, Dinney and colleagues[45] showed that systemic treatment with interferon-α blocked expression of bFGF and was associated with decreased microvessel density and reduced tumor growth. An interferon-resistant tumor cell line and immune-deficient animals were used in their studies to eliminate the effects of interferon on cellular proliferation and the immune system and a primary effect on angiogenesis was implicated. Bladder cancer, the fifth most common human malignancy, can present as superficial tumors confined to the urothelial lining that have exceptionally high rates of local recurrence or as muscle-invasive disease that is often associated with tumor progression and metastasis.[46] Novel treat-

ment modalities, such as antiangiogenic approaches, are greatly needed for this malignancy. The possibility of using TSP-1 peptide mimetics for the prevention or treatment of bladder cancer is particularly appealing since TSP-1 is the primary natural inhibitor of angiogenesis in normal bladder tissue and its loss underlies the switch to angiogenesis that occurs during bladder tumorigenesis.[18] Excretion of such peptides into the urine, allowing for increased concentration and prolonged exposure to the urothelium, may also improve therapeutic efficacy against this particular malignancy. However, the ability of peptide mimetics to block the metastatic process, as shown in the B16F10 melanoma model and the mechanism of action, which targets the "final common pathway" by activating apoptosis in endothelial cells, suggest a more general utility against a variety of malignancies or other angiogenic diseases.

Several large proteins with potent antiangiogenic activity are now in early-stage clinical trials.[47–50] However, use of whole, unmodified protein molecules entails a number of problems, including costly purification, short half-life, conformational instability and possible immunogenic reactions or side effects from other biologic activities.[24,25,51,52] Our peptides retain the antiangiogenic function of TSP-1 while avoiding the potentially deleterious effects associated with its other functional domains. Here, we describe the use of such receptor-targeted short antiangiogenic peptides and demonstrate their antitumor efficacy. These peptide mimetics will join the new generation of small, stable and cost-effective compounds that may eventually play an important role in the treatment of angiogenesis-dependent diseases such as arthritis, diabetic retinopathy and cancer. Other promising compounds in this group include the RGD-containing peptides and their synthetic mimetics, which block essential interactions between the $\alpha_v\beta_3$ integrins expressed by endothelial cells and matrix components,[53] and the small synthetic inhibitors of tyrosine kinases, which transmit activation signals from VEGF and other important endothelial cell growth factors.[54,55] Our peptides are unique in that they activate pathways directly leading to endothelial cell inhibition and apoptosis, rather than achieving this indirectly by blocking survival signals.[21]

While our peptides blocked tumor growth by 70–80%, this inhibition was not complete and relatively high doses were required, emphasizing the need for additional refinement to optimize results with this therapeutic approach. The peptide structure of DI-TSP is currently being modified in search of even more potent peptides or those with more favorable pharmacokinetic properties. The crystalline structure of DI-TSP is also under study and this may facilitate the design of small synthetic compounds, recapitulating the active sites of DI-TSP. These synthetic compounds could potentially provide a number of distinct advantages, such as increased potency and stability, more rapid and cost-effective chemical synthesis and oral administration. In addition, the efficacy of the peptides could be enhanced by upregulating the expression of CD36 on endothelial cells and we have demonstrated that this can be accomplished by treatment with the noninflammatory cyclopentenone prostaglandins.[56] *In vitro,* this family of prostaglandins, which activates the peroxisome proliferator–activated receptor-γ, upregulated CD36 expression on endothelial cells and markedly enhanced their response to the inhibitory effects DI-TSP; synergy between cyclopentenone prostaglandins and DI-TSP was also observed *in vivo* in the corneal neovascularization assay.[56] Finally, the efficacy of DI-TSP treatment could also be enhanced by combination therapy with other antiangiogenic agents or with cytotoxic treatments such as chemotherapy or radiation therapy. Several reports substantiate additive or synergistic effects in preclinical models when such therapeutic modalities are used together in a multimodal approach to the treatment of cancer.[8,9] Our peptides should serve as excellent lead compounds in the further development of such integrated and optimized antiangiogenic strategies.

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